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(71) Applicant (*for all designated States except US*): UNIVERSITY OF PENNSYLVANIA [US/US]; 3700 Market Street, Suite 300, Philadelphia, PA 19104-3147 (US).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): DIAMOND, Scott, L. [US/US]; 610 Yale Road, Bala Cynwyd, PA 19004 (US).

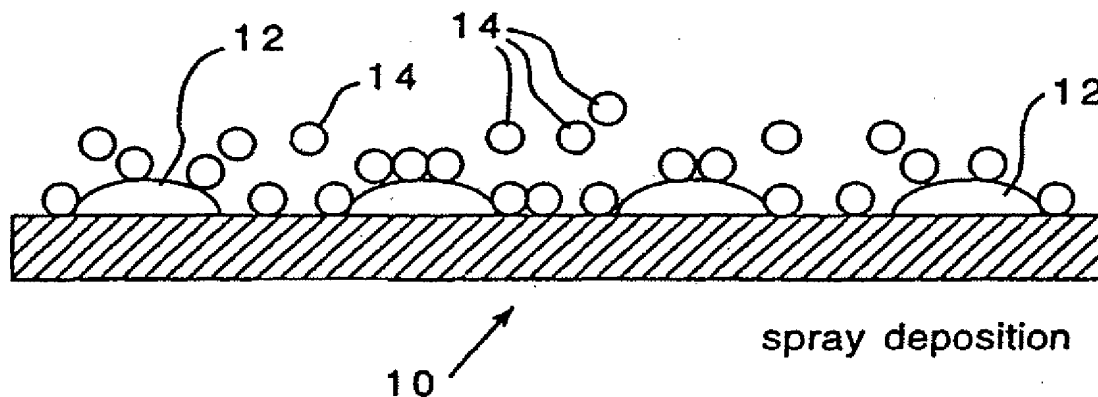
(74) Agents: JOHNSON, Barbara, E. et al.; Webb Ziesenheim Logsdon, Orkin & Hanson, 700 Koppers Building, 436 Seventh Avenue, Pittsburgh, PA 15219-1818 (US).

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(54) Title: PEPTIDE OR PROTEIN MICROASSAY METHOD AND APPARATUS



(57) Abstract: A peptide or protein microassay method and apparatus in which a wide variety of chromogenic or fluorogenic peptide or protein substrates of interest are individually suspended or dissolved in a hydrophilic carrier, with aliquots of each substrate being deposited in an array or microarray of reaction loci, or "dots." Each dot, therefore, provides an individual reaction vessel containing the peptide or protein of interest, to which a biological sample may be applied for assay purposes. The sample is applied to the array or microarray of dots by one of a variety of focused sample application techniques, including aerosolizing or misting of the sample, or target application of the sample, onto each dot without creating fluid channels between the dots which would cause cross-contamination.

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**PEPTIDE OR PROTEIN MICROASSAY  
METHOD AND APPARATUS**  
BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The invention relates to a microassay chip and method for analysis by means of peptides or proteins for use in biological research and biomedical diagnosis.

2. Description of the Related Art

[0002] In biological research, biomedicine and industrial applications, large scale genomic evaluation for the detection of specific genes or DNA sequences within a genome, specific gene mutation such as single nucleotide polymorphisms (SNP), and mRNA species are well-established methodologies. These methodologies utilize DNA chips and microarrays on which specific nucleic acid sequences are either synthesized or deposited at individual highly localized positions on an array. These arrays containing the nucleic acid sequences find support on solids such as silicon or glass, or materials such as nylon membranes. The sequences can exist in the array on the order of  $10^3$  or  $10^4$  individual microsamples because individual "dots" or "pixels" have sub-millimeter characteristic lengths. While these chips have many applications for detecting the presence of and identifying genes in a genome (genotyping) or evaluating patterns of gene regulation (mRNA profiling) in cellular and tissue systems, these nucleic acid-based systems provide no information about the activity or regulation of the gene product, i.e., the synthesized protein.

[0003] Currently, DNA chips and microarrays allow genotyping and expression profiling, without rendering information about the activities of enzymes which can be regulated by phosphorylation or cleavage states. Protein chips to date have involved the capture of proteins to immobilized DNA sequences or libraries of immobilized peptides, antibodies or proteins. The three major formats for protein arrays employ plain glass slides,

three-dimensional gel pad chips ("matrix" chips) or nanowell chips. None of these formats utilizes soluble substrates to identify numerous enzymes in a simple assay, however.

[0004] Proteomic methods typically utilize two-dimensional electrophoresis gels to separate proteins, followed by enzyme digest mapping and/or mass spectrometry to characterize relevant individual proteins in the gel. Neither DNA chips nor two-dimensional electrophoresis provide information about the activity of the protein or its reaction kinetics. For example, an enzyme may require phosphorylation or dephosphorylation in order to have full activity, and prior chip technologies do not provide this information.

[0005] Presently, enzyme activity can be measured by incubation of the enzyme with chromogenic substrates whose cleavage products become intensely colored and absorb light at a particular wavelength. Alternatively, the substrate may be a fluorogenic substrate whose cleavage results in leaving groups that are intensely fluorescent when excited at a particular wavelength (8-EX). Emission wavelengths of the leaving groups may span 10 to 20 nm above and below the maximum 8-EM. This prevents the use of more than two or three different fluorogenic substrates in a single sample to assay for three different enzymatic activities since the emission of each substrate may have significant overlap with the emission of the other substrates. Broad band emission results in color cross-talk and can render false signals. Thus, it is not possible to add 10 to 100 different fluorogenic substrates to a single fluid sample because the emissions would overlap severely. These reactions are typically monitored in cuvettes in a fluorimeter or plate-reader with working volumes of 0.2 to 3 ml. Thus, significant dilution of the sample occurs.

[0006] The evaluation of various proteins and/or enzymes within a small biological sample (1.0 to 100 nL) would be useful in analyzing the activity of those proteins and/or enzymes in a number of fields of study. In the field of cell biology and cancer, the timing of cell division is regulated by numerous cyclin-dependent kinases (cdk), cAMP-dependent

kinases (PKA), cGMP-dependent kinases (PKG), and calcium-dependent protein kinases (PKC), tyrosine kinases, and tyrosine phosphatases. In the field of hematology, the function of blood is regulated by various coagulation factors, complement factors and fibrinolytic factors which are proteases and inhibitors necessary for thrombotic and thrombolytic mechanisms. During apoptosis (programmed cell death) various caspases are critical to the cascade of events. Similarly, neutrophil activation during sepsis, thrombosis or infection is coordinated with release of elastases, proteases or other enzymes. Tumor invasion and intimal hyperplasia can involve the activity of metal metalloproteases (MMPs) and tissue inhibitor of metalloproteases (TIMPs). Various viral activities (e.g., proteases) would be suitable for detection of drug screening of protease inhibitors.

[0007] Notwithstanding prior art developments in the areas of peptide and protein chips, therefore, the need for peptide or protein microarrays in diagnostic, prognostic and clinical medicine is large, and largely unmet. Prior art chips do not exist in which a great variety of suspended or soluble chromogenic or fluorogenic substrates may be simply deposited in an array on a support surface, with simple application of the sample fluid thereto for evaluation. At this writing, there are no known peptide or protein chips which can be directly fabricated using a standard contacting or non-contacting microarrayer, for example. Liquid layer sample applications over unbound substrate molecules would be considered unthinkable, moreover, due to the inevitable cross-contamination such liquid sample layers would engender. As a result, a need remains for a simple, effective and inexpensive peptide or protein array or microarray system which provides an easily fabricated chip using standard microarrayer equipment, which provides a system in which elaborate compensations such as peptide or protein binding, or quenching layers are unnecessary, and to which sample may be simply and easily applied. Also, the need likewise persists for a system which can rapidly

deliver small liquid samples to individual reactant positions of an array or microarray without cross-contamination among the reactant positions.

### SUMMARY OF THE INVENTION

[0008] In order to meet this need, the present invention is a peptide or protein microassay method and apparatus in which a wide variety of chromogenic or fluorogenic peptide or protein substrates of interest are individually suspended or dissolved in a hydrophilic carrier, with aliquots of each substrate being deposited in an array or microarray of reaction loci, or "dots." Each dot, therefore, provides an individual reaction vessel containing the peptide or protein of interest to which a biological sample may be applied for assay purposes. The sample is applied to the array or microarray of dots by one of a variety of focused sample application techniques, including aerosolizing or misting of the sample, or target application of the sample, onto each dot without creating fluid channels between the dots which would cause cross-contamination. In a first embodiment of the present invention, the sample is misted or aerosolized, and the application of such an aerosolized sample to the dots results in the sample's being absorbed by the individual dots while any excess sample droplets between the dots either tend to migrate toward and be absorbed by the nearest dot, or evaporate, leaving each dot as a discrete reaction chamber without fluid reactant connection to any other dot. Known scanning and database creation techniques may be used to analyze reaction indicators present or absent in the arrays of dots.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0009] Fig. 1 is a partial perspective view of an array according to the present invention;

[0010] Figs. 2A, 2B and 2C are side elevational views of arraying, aerosol sample deposition and substrate conversion, respectively;

- [0011] Figs. 3A and 3B are schematic diagrams of peptide or protein microarrays before and after sample application;
- [0012] Fig. 4 is a sectional view of an ultrasonic misting device;
- [0013] Fig. 5 is a functional diagram of the assay apparatus;
- [0014] Fig. 6 is an array of carrier solvent (glycerol) microdots;
- [0015] Fig. 7 is a prespray array of carrier solvent (glycerol) microdots;
- [0016] Fig. 8 is an array of microdots sprayed with water-based sample;
- [0017] Fig. 9 is a proximal view of a sprayed microdot array after mist evaporation;
- [0018] Fig. 10 is an activated microdot array;
- [0019] Fig. 11 shows fusion of water droplets containing sample with glycerol microdot;
- [0020] Fig. 12 shows detection of thrombin activity by microdot assay;
- [0021] Figs. 13A, 13B, 13C, and 13D show microfluidics technology for reagent delivery to individual reaction compartments;
- [0022] Fig. 14 shows delivery of molecules to reaction spots using spray generated from an ultrasonic nozzle;
- [0023] Fig. 15 shows generation of ultrafine mist using ultrasound transducer and non-contacting chamber;
- [0024] Fig. 16 shows delivery of molecules to reactive spots using small droplet spray generated by an ultrasound transducer and non-contacting chamber;
- [0025] Fig. 17 shows that adjacent reaction spots do not cross-contaminate after spray delivery of mist;
- [0026] Fig. 18 shows a microarray assay of purified enzymes and human plasma;
- [0027] Fig. 19 is a microarray of caspase substrate;
- [0028] Fig. 20 is an activated caspase microarray;

- [0029] Fig. 21 shows mist delivered by an assay system;
- [0030] Fig. 22 shows fluorescent mist delivered to microarray; and
- [0031] Fig. 23 shows capture of mist on microarray using an electrostatic charge.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0032] The present invention is a peptide or protein microassay method and apparatus in which a wide variety of chromogenic or fluorogenic peptide or protein substrates of interest are individually suspended or dissolved in a hydrophilic carrier, with aliquots of each substrate being deposited in an array or microarray of reaction loci, or "dots." Each dot, therefore, provides an individual reaction vessel containing the peptide or protein of interest to which a biological sample may be applied for assay purposes. The sample is applied to the array or microarray of dots by one of a variety of focussed sample application techniques, including aerosolizing or misting of the sample, or target application of the sample, onto each dot without creating fluid channels between the dots which would cause cross-contamination. In a first embodiment of the present invention, the sample is misted or aerosolized, and the application of such an aerosolized sample to the dots results in the sample's being absorbed by the individual dots while any excess sample droplets between the dots either tend to migrate toward and be absorbed by the nearest dot, or evaporate, leaving each dot as a discrete reaction chamber without fluid connection to any other dot. Known scanning and database creation techniques may be used to analyze reaction indicia present or absent in the arrays of dots.

[0033] Generally, this invention can be applied to areas of biotechnology and biomedical research. More specifically, this invention can be used to study enzyme activities, as well as cofactors, inhibitors and activators of enzymes. In one application, the assay may be used in drug research, namely drug discovery, by screening the effect of large combinatorial libraries of compounds on activities of generally between ten up to thousands

of enzymes, or drug interaction in blood chemistry, matrix metalloproteases, angiogenesis, tyrosine phosphoprotein phosphatases, or apoptosis regulation. The assay can be applied to genomics or proteomics research, in particular, epigenetic regulation of enzyme systems. In a further application, the assay can be used to research signal transduction pathways, such as kinase and phosphatases in gene regulation. The assay may also be used for structural and functional research of combinatorial studies involving point mutations on enzyme substrate specificity. The assay may be applied to blood research, namely, coagulation diagnostics and thrombolytic research, or the assay can be applied to viral research and diagnostics of viral proteases and processing activities. The assay can thus be employed throughout various fields of biological research due to its simplicity and versatility. As mentioned above, the assay is suitable for use with existing scanning technologies in place for genomic studies.

[0034] In the above and ensuing description, the following terms may be understood as follows. A reaction loci is generally an adherent non-spreading volume of fluid on a solid surface. A reaction spot can be referred to as "spot", "dot", "reaction zone", "reaction center", "microdot", or "microassay." A chip is a planar surface containing non-spreading reaction dots. Chips can also be referred to as a "glass slide", "slide", "surface", "solid substrate", "bioreaction microarray", "bioreaction chip", and "bioreaction slide." Spray refers to the delivery of an aerosol of liquid sample to a solid surface containing reaction spots. Spray can also be referred to as a "mist", "aerosol", "atomized mist", "droplet/s" or "nebulized mist."

[0035] The present assay generally comprises microreactions in a liquid phase which are created by applying small volumes of a fluid mixture of a peptide or protein substrate, a hydrophilic carrier solvent and a volatile solvent to a nonporous surface, whereby evaporation of the volatile solvent results in highly localized long-lasting liquid or semi-solid dot or microdot residues of substrate in a hydrophilic carrier solvent. The substrate is fluorogenic or chromogenic to enable analysis of the reaction, if any, within the hydrophilic



carrier after the sample is applied. The nonporous surfaces for delivery of the fluid mixture can include silicon, glass, silica, quartz, polystyrene or other nonporous polymeric membranes. Overall, the components of the assay are usually combined and applied via a computer-controlled application system, and microreactions are monitored via a computer-based scanning and database producing system.

[0036] Particularly, when the arrays involved are microarrays, the presence of the volatile solvent facilitates fluid creation of the microdot by reducing the overall viscosity of the formative fluid admixture. The volatile solvent generally has the ability to evaporate and suitable volatile solvents include, without limitation, dimethylsulfoxide (DMSO); chloroform; acetone; acetic acid; water; an alcohol such as methanol, ethanol or propanol; ethyl ether or alkane. After application of the fluid admixture to a nonporous surface, the volatile solvent evaporates, leaving microdots containing hydrophilic carrier solvent and the suspended or dissolved chromogenic or fluorogenic substrate(s). These constituents remain in a liquid or semi-solid state without crystallization or precipitation of the substrate(s).

[0037] At the time of a sample application, the hydrophilic carrier suspends or dissolves the substrate(s) to maximize the bioreaction potential with later applied biological samples. The hydrophilic carrier generally possesses the following characteristics: miscibility with the volatile solvent; miscibility with water; miscibility with aqueous biological fluids; suitability for maintaining a stable solution or suspension of fluorogenic or chromogenic substrate(s) at high concentrations; moderate viscosity between 1 centipoise and 10,000 centipoise; compatibility with biological molecules such as nucleic acids, peptides, proteins, and sugars; suitable fluidity for movement into and out of microcapillary devices such as the hollow tips of microarray pins or microsyringes used for arraying; a specific contact angle sufficient to form a stable finite lens where the bioreaction fluid in the spot after arraying does not spread (contact angle  $> 0$  is required); a specific contact angle low enough

to form a stable adherent lens that does not have too low of adhesion such that the spot has limited adhesion and can roll on the substrate (contact angle  $< 90$  is required); and low volatility such that the reaction zone does not evaporate. Glycerol (1,2,3-propanetriol) is an example of such a fluid that possesses all of these characteristics. Other examples of the hydrophilic carrier solvent include a polyalcohol such as 1,2-ethanediol or 2,3-butanediol. In addition, the carrier solvent may contain viscosity enhancers such as dextran, pluronic acid, carbohydrates of the pentose, ribose or hexose families or related polysaccharides or polyethylene glycol polymers.

[0038] The microdots are generally applied to the nonporous surface in a microarray configuration. The final volume of the microdot, after evaporation of the volatile solvent, ranges from about 1 nL for a 10  $\mu\text{m}$  diameter microdot to about 1 to 10 nL for a 100  $\mu\text{m}$  dot. Microdots can be applied through fluid handling methods of direct positive displacement pumping. Alternatively, the microdot is applied through "arraying", whereby computer controlled metal, glass or plastic tips pick up droplets of fluid from a reservoir by capillary action and make contact with the solid surface, or by laser printing or jet printing techniques. Arraying is accomplished by using well-established pin technologies (i.e., Telechem Pins, GeneMachine arrayer). The separation distance between microdots ranges from 50 to 1000  $\mu\text{m}$ . Delivery of 1 to 10 nL of formulation is sufficient to create a microdot.

[0039] After creating a high density array of microdots, each of which contains a specific fluorogenic or chromogenic reporter substrate, as well as other possible reaction modifiers, a small sample of biological fluid is applied to the microdots. Each microdot is inoculated with sample by application of the biological fluid, generally through deposition of a fine mist on the biochip. The mist is applied in a manner that does not form a wetting film and never bridges two adjacent glycerol droplets. In other words, the application of the aerosolized sample to the dots results in the sample's being absorbed by the individual dots

while any excess sample droplets between the dots either tend to migrate toward and be absorbed by the nearest dot, or evaporate, leaving each dot as a discrete reaction chamber without fluid reactant connection to any other dot. Delivery of the biological fluid containing a corresponding relevant enzyme will cause reaction and concomitant activation of the chromogenic or fluorogenic substrate in each glycerol droplet. In other words, enzyme or chemical constituents of the biological fluid lead to the activation or antagonism of the activation of the fluorogenic substrate in the microdot to produce a fluorogenic or chromogenic signal readable by epifluorescence or confocal scanning, direct imaging or light absorption. An individual chip can be configured to report the activity of numerous proteases, kinases, phosphatases, oxidoreductases, lipases and inhibitors or activators of these enzymes, each within an individual dot or microdot loci of each reaction of interest.

[0040] It should be borne in mind that the present peptide and protein chips are considerably simpler than most if not all prior art arrangements which include means for physically adsorbing or binding peptides or proteins directly to the glass slide or chip, or which contain multiple components including but not limited to quenching overlayers, gel pads or other features more complex than the present reaction loci. Constituents inconsistent with the practice of the present invention would be anything which would interfere with the hydrophilic carrier's providing a discrete reaction vessel containing the peptide or protein of interest and any other constituents designed to facilitate sample absorption, reaction and reaction detection.

[0041] The invention is further illustrated in the accompanying Figs. 1-5.

[0042] Referring now to Fig. 1, a biochip 10 has arrayed thereon a plurality of reaction loci 12, over which are applied the aerosolized or misted or ink-jet printed sample droplets 14. The vertical arrow illustrates vertical deposition of the sample droplets 14.

[0043] Figs. 2A, 2B and 2C are side elevational views of arraying, aerosol sample deposition and substrate conversion, respectively, according to the present invention. In Fig. 2A, a microarrayer tip 16 is shown depositing the reaction loci 12 onto the biochip 10. In Fig. 2B, the aerosolized or misted sample droplets 14 are shown in the process of deposition onto the reaction loci 12. In Fig. 2C, the sample droplets 14 have either absorbed into the reaction loci 12 or have evaporated and disappeared completely from the biochip 10. In Fig. 2C, individual reaction loci 12 can generate color or fluorescence as a result of substrate conversion.

[0044] Fig. 3A is a schematic diagram showing a blanket sample application over all the reaction loci 12 of the biochip 10 in a square or rectangular pattern 18, whereas the reactant of Fig. 3B reposes solely within the confines of the reaction loci 12 of the biochip 10. The result shown in Fig. 3B can occur through various mechanisms including but not limited to the blanket sample application may be of an aerosol or mist which evaporates from between the reaction loci 12; or the sample may be targeted for application to the reaction loci 12, such as by a laser printer or ink-jet printer; or the sample may be applied through a mask or template which blocks sample application from any area other than the reaction loci 12.

[0045] Fig. 4 illustrates, in section, a nebulizer 20 containing an ultrasonic generator (transducer) 21 filled with a liquid 22 adapted to receive a container 25 having a sample 26 therein, whereupon energization of the sample 26 by the transducer 21, the aerosol mist made from the sample 26 may exit the nozzle 32 for deposition on the biochip 10. Optionally, a carrier gas 24 enters the container via inlet 23, which carrier gas 24 helps to displace the aerosolized sample 26 for deposition onto the biochip 10. For the purpose of Fig. 4, it should be borne in mind that the biochip 10 is inverted compared to the biochip position shown in the remaining Figures. The nebulizer 20 also contains optional placement guides 28 for

holding the container 25 in position, as well as a cap 30 for the container 25 through which the nozzle 32 extends as shown.

[0046] Fig. 5 is a schematic illustrating the juxtaposition of various components of the present assay system as positioned adjacent the biochip 10, the reaction loci 12 and the sample droplet(s) 14. These components may include, without limitation, a printhead for sample application, an xyz positioner, a power supply, a controller, and means for providing excitation light and detecting emission signal, among other components. More particularly, major components of the assay system (apparatus) include a set of operating instructions resident in computer software that sends via serial or parallel port signals to start, to stop, to establish operating set point, and to control the subcomponents of the assay device, whereby each subcomponent may have an internal or external standing controller or driver. The subcomponents of the device include the following: multiple positive displacement microsyringe pumps; aerosol generating devices, such as pressure nozzles, ultrasonic nozzles, ink-jet printheads, position-actuated ink-jet printheads, surface-actuated ink-jet printheads, fluid contacting or fluid non-contacting ultrasonic transducers; gas flow meter/controller; xy positioner system; and an exhaust/filtration fan.

[0047] Aerosolized sample application may be facilitated through the use of computer controlled microsyringes. The computer controlled microsyringes are used for timed sample delivery at constant low rate, and each positive displacement syringe can hold from 1.0  $\mu\text{L}$  to 1000  $\mu\text{L}$  of biological sample, whereby the sample may be organic molecules, fluorogenic molecules, peptides, proteins, lipids, dilute solutions of polymers, liquid with coated microbeads, sample buffers, wash buffer biological cells and cell fractions. Each positive displacement pump delivers the sample to the site of aerosol generation. The positive displacement pump may be maintained in an environment that is refrigerated, at room

temperature or heated. The positive displacement pump is controlled by a device that receives signals from a computer.

[0048] There are various ways to aerosolize the sample for application to the present system. One system relies on pressure nozzles, whereby the fluid sample to be aerosolized is pumped at high pressure through small orifice nozzles to generate an aerosol spray. In some nozzles, the fluid is carried by a pressurized inert carrier gas such as nitrogen, helium, air or oxygen. High velocity gas streams can be used to pull the fluid sample, by Bernoulli effect, into the nozzle and through the nozzle orifice. The carrier gas next delivers the aerosol to the biochip for deposition on the individual reaction dots. Nozzles may have internal components to facilitate aerosol formation. In one example, a small biological sample (0.1 to 1 ml) is pulled into a nozzle by pressurized gas flow (5 to 30 psig nitrogen) through a 250 micrometer orifice with a 210 micrometer inner needle to facilitate atomization.

[0049] Alternatively, spray aerosolization results from the use of ultrasonic nozzles which give low volumetric flow rate and uniform low velocity (under 10 cm/sec) mists. Ultrasonic standing waves within the nozzle cause atomization of the fluid at the tip of the nozzle. Low flow rates from 0.01 to 1000  $\mu\text{L}/\text{sec}$  can be achieved by micropump delivery of sample into the nozzle body that contains a piezoelectric ultrasound transducer operating between 25 kHz to 240 kHz to create mists with average drop sizes from 5 to 15 micrometers in diameter. Energizing of the piezoelectric ultrasound transducer can utilize low wattage (from 0.1 to 25 watts) to avoid unwanted heating of the sample. For instance, a biological sample with the viscosity of 0.01 poise is pumped by a microsyringe pump at a flow rate of 0.1 to 1  $\mu\text{L}/\text{sec}$  into an ultrasonic nozzle operating at 120 kHz (0.1 to 1 watt). Carrier gas streams external to the nozzle help to direct the mist to the bioreaction chip surface.

[0050] Another means of creating aerosolized sample relies on a contacting ultrasonic nebulizer where a fluid is placed in a well, the bottom of which contains an ultrasonic

piezoelectric transducer. The transducer is operated at 1.0 to 3.0 MHz. The high frequency vibration at the top surface of the liquid in the sample chamber facilitates the formation of an atomized or nebulized cloud of fluid droplets. The action of nebulization causes the nebulized aerosol to rise from the chamber toward a bioreaction chip surface suspended atop the chamber. Additionally, a carrier gas can be introduced into the nebulizing chamber upwardly to displace the cloud. Alternatively, a carrier gas can be passed over the nebulizing chamber to pull the nebulized aerosol into the carrier stream by Bernoulli effect. The carrier gas is directed toward the samples to receive the aerosol. Atomized fluid particle diameter ( $d$ ) is related by the surface tension ( $T$ ), density ( $\rho$ ), and the frequency ( $f$ ) by the following approximate equation of:  $d \sim (T/\rho f^3)^{1/3}$ . For example, nebulization of water ( $T = 0.0729$  N/m,  $f = 2.4$  MHz) produces 1.7 micrometer mist droplets.

[0051] A further means of aerosol generation relies on a non-contacting ultrasonic nebulizer, in which a fluid to be delivered is placed in a tube that has a thin walled plastic bottom suitable for transmission of ultrasonic waves. The tube is placed in a conducting fluid that is in contact with the ultrasonic transducer and the fluid sample to be aerosolized, therefore, never comes in contact with the ultrasonic transducer *per se*. The transducer is generally operated between 1.0 to 3.0 MHz, and the high frequency vibration at the top surface of the liquid in the sample tube facilitates the formation of an atomized or nebulized cloud of fluid droplets in the sample tube. The action of nebulization can cause the nebulized aerosol to rise in the chamber and a carrier gas is delivered into the sample tube to displace the mist (optionally through a coarse collecting filter to remove large droplets) toward the bioreaction chip. Nebulized fluid samples prepared with this non-contacting ultrasonic nebulizer will generally have droplet sizes ranging from 1 to 25 micrometers.

[0052] Finally, ink-jet Piezo-printing, where delivery of a biological fluid into the printing head that exploits non-heating ink-jet technology may be used to propel the sample

fluid toward a bioreaction spot. In the first approach of Piezo-printing, the printhead continually "prints" the biological fluid over the entire slide in a raster pattern of printing without discrimination of its position over the reaction spot or over glass. In this non-specific mode of operation, the ink-jet functions as a spray head, albeit one that creates a very narrow zone of spray and one that requires xy positioning with time over the bioreaction slide in order to actuate the entire slide. In this approach, the novel reaction zone isolation of the formulation fluid (glycerol) and the ability to print without forming a wetting film or a continuous layer of printed biological fluid allows reaction compartmentalization without spot-to-spot cross-bridging even though the entire surface is printed.

[0053] In the second approach of Piezo-printing, the printhead delivers biological fluid at discrete times. The delivery of a droplet of fluid to the reaction spot is triggered (1) by known information about its position relative to the known position of the reaction spots or (2) by sensing a property of the glycerol spot which triggers the delivery of fluid via ink-jet printing to the reaction spot. For example, a bifurcated fiber optic could excite a fluorescent dye in the reaction spot whose emission is transmitted via the fiber optic cable to an emission filter and a photomultiplier tube. The output of the photomultiplier tube is amplified, digitized and triggers the printing event. Alternatively, any optical property of the reaction spot can be sensed to trigger the activation of the bioreaction zones.

[0054] With any of the above-described sample application techniques or their equivalents, masking devices may be used to encourage sample application directly to the microdot. A masking device, such as a template or pattern, may be temporarily placed over dot array or microarray, or masking materials may be incorporated into the sample application equipment. Masking should be understood as optional to the present invention.

[0055] The assay is preferably contained within a computer-controlled gas flow rate metering system. A supply of inert carrier gas is supplied at high pressure with a regulator to



control system pressure to under 50 psig. Typical pressure settings are between 5 to 15 psig. The gas flow uses the Bernoulli effect to put the aerosol stream toward the target slide to be activated. The carrier gas can be any of the following: air, oxygen, nitrogen, helium or argon. The gas flow rate can be between 0.1 and 5 L/min to help direct the aerosol from various nozzles. Alternatively, gas flow may be used to carry the liquid sample into the pressure nozzle by Bernoulli effect. It should be understood, however, that the use of these carrier gases is optional to the present method and apparatus.

[0056] For sample application and assay monitoring, the assay system preferably includes a computer controlled xy positioner with an independent x and y axis of movement. The xy positioner translates a removable stage that contains an accessible housing for assay chips ready for microdot application and assay activation. The removable stage allows transfer from the arrayer into the sprayer, and subsequently into the incubator while avoiding hands on contact between the operator and the individual slides. The xy positioner has a travel range from 0.1 m by 0.1 m up to 10 m by 10 m. Smaller distances of the xy positioner stage travel can be achieved by a linear stepper motor or servomotor. Longer distances can be achieved by motorized belt drive assemblies with motors that are controlled by individual drivers that receive driving signals from the main operating computer. A velocity of 0.1 to 20 in/sec can be achieved for rapid translation of slides under the aerosol.

[0057] Specifically, the assay method may involve dissolving a fluorogenic substrate in a volume of DMSO at 10  $\mu$ M to 1 M concentration. A 0.1-1 volume of glycerol is then mixed with the substrate DMSO solution. This solution is delivered to a glass substrate by micromanipulation of a small diameter metal or plastic probe. The microdot can release its volatile DMSO component at room temperature. A mask is placed over the glass substrate to cover at least a portion of the areas among the microdots. A microspray of biological fluid is delivered through the mask to the microdots on the surface. The microdot array is incubated

from 0 to 180 mins at 4-37°C in 0 to 100% humidity. Conversion of substrate is detected in the microdot by excitation (350-400 nm) and emission above 420 nm using any available detection means.

[0058] It should be borne in mind that, in the practice of the present invention, the substrate need not start out as chromogenic or fluorogenic if it can be made so later in the process of assaying the sample. A second spray containing a reporter substrate is described to this end in Example 1 below.

### EXAMPLES

#### Example 1:

[0059] A microarray of glycerol loci is created in which each glycerol spot (50 to 250 micrometers in diameter with a 50 to 500 micrometer space between spots) contains a single molecular species "A" such as a protein or protein fragment; a synthetic peptide; a small organic molecule; a nucleic acid sequence or a synthetic polymer. This species resides at a concentration between 10 picomolar and 10 millimolar within the spot. A 1"x3" glass slide is configured to yield over 10 cm<sup>2</sup> of arrayed glycerol loci at 100 to 1000 spots/cm<sup>2</sup>.

[0060] An aerosol containing an enzyme (at 1 picomolar to 10 millimolar) is delivered to the array, whereby droplets of the enzyme fuse with the reaction spots while droplets that hit the slide quickly evaporate. For a period of time, the enzyme is allowed to come into binding equilibrium with the chemical species, typically 1 to 60 mins. A second spray contains a reporter system to detect the enzyme activity. Such a reporter system may consist of a fluorogenic substrate; a chromogenic substrate; a cofactor along with a detection system for cofactor conversion; or a cofactor and a substrate, whereby cofactor conversion produces a detectable signal. This reporter system can be delivered at concentrations from 10 nM to 100 mM with a fluorogenic substrate.

[0061] If the molecular species "A" binds the active site or an allosteric position of the enzyme "E", then the activation of the reporter system (e.g., conversion of a fluorogenic substrate) is prevented. Reaction zones that produce no reporter signal or reduced reporter signal are then identified as inhibitors of the enzyme. The inhibitor effect may be due to the molecular species "A" acting as a competitive inhibitor; a suicide inhibitor; a noncompetitive inhibitor; an allosteric modulator; a complexation agent against the substrate; an antagonist of cofactor binding; a complexation agent against the cofactor or an uncompetitive inhibitor.

Example 2:

[0062] A microarray is set up to analyze binding events between antigens and antibodies. Initially, a polyclonal or monoclonal antibody is chemically linked to a colloidal object, such as colloidal gold (10 nM to 200 nM) or latex bead (10 nM to 200 nM). The latex beads with covalently linked antibodies are added to glycerol to create a suspension prepared for microarraying. The glycerol may also contain an unlinked polyclonal or monoclonal antibody against a particular antigen of detection. The concentration of the antibody covalently linked to the bead may range from 1 to 10,000 sites per square micron of the bead. The concentration of the free antibody in solution can range from 0 to 100  $\mu$ M.

[0063] An array of spots is generated (spot size of 50 to 250 micrometer diameter with a 50 to 500 micrometer space between spots). Each spot has a unique composition whereby colloidal objects with linked antibody are maintained at a single concentration and the quenching free antibody is maintained at increasing concentration in a series of spots. A biological sample containing the antigen of detection is delivered via spray mechanism to the array. In each reaction spot, the quenching antibody binds the antigen (a faster reaction since the free antibodies have greater Brownian motion in comparison to the antibodies bound to the beads). If the concentration of the antigen exceeds the concentration of the quenching antibody, then free antigen remains and is available for binding to the beads. Beads will

undergo antigen-mediated agglutination. The agglutinated colloidal gold or latex beads can be quantified by computer-assisted imaging of the aggregates, enhanced light scattering by aggregates or reduced light transmission by aggregates. Through use of known increasing concentrations of quenching antibodies in a series of spots, the concentration of antigen can be estimated to be equivalent to the minimum concentration of quenching antibody preventing agglutination.

Example 3:

[0064] Detection of an antigen in a biological fluid is accomplished with a non-fluorescent species of unknown concentration. A series of glycerol droplets is arrayed with each containing a fluorescent species with a binding site. The reporter molecular would be under 30 kDa. Such a reporter molecule would be a GFP-ScFv chimeric protein where the single chain Fv fragment has high binding affinity for the antigen to be detected.

[0065] The antigen to be detected is sprayed onto the array surface and is sufficiently large (greater than 5 kDa) causing a significant change in the total molecular weight of the detector molecule/antigen complex. The detector molecule/antigen complex is analyzed by the polarization of the fluorescence emission of the complex which is greater than that of the reporter molecule alone when the system is excited with polarized light.

Example 4:

[0066] Well characterized for over 30 years, serine proteases of the coagulation pathway in plasma were selected as a system in which to monitor the delivery of complex enzyme fluids to the microassay. Fluorogenic substrates in DMSO/glycerol were assayed to a glass slide.

[0067] After spray application, thrombin caused a 65-fold increase in fluorescence activity after conversion of the boc-VPR-MCA substrate. When deposited on the array, pure plasmin (1  $\mu$ M) gave an intense signal for the boc-VLK-MCA substrate (25-fold above

background) with detectable activity on the thrombin and kallikrein substrates. Plasmin has been reported to have activity on boc-VPR-MCA and Z-FR-MCA with no detectable activity on tPA and factor Xa substrates (Morita, 1977). Aerosol deposition of pure tPA (10  $\mu$ M) to a sensing array caused significant cleavage of the tPA, thrombin and Xa substrates, resulting in fluorescence that was 18- to 20-fold above background.

[0068] The microarray detected the modest activation of the coagulation pathway in recalcified diluted plasma due to low level factor Xa and thrombin activity (Butenas, 1997; Rand, 1996). Since corn trypsin inhibitor was not present to inhibit factor XIIa (Rand, 1996), moderate activation of the contact system and kallikrein generation was detected by conversion of the kallikrein substrate. Recalcified citrated plasma does not generate plasmin in excess of the  $\nabla$ 2-antiplasmin and  $\nabla$ 2-macroglobulin consistent with the lack of conversion of the plasmin substrate. The microarray revealed that the conversion of kallikrein substrate occurred from the contact activation of prekallikrein to kallikrein, not from non-specific Z-FR-MCA substrate cleavage by plasmin.

[0069] Addition of plasmin (0.43  $\mu$ M final concentration) to the diluted recalcified citrated plasma overcame inhibitory concentrations of  $\nabla$ 2-antiplasmin and  $\nabla$ 2-macroglobulin. This plasmin activity was detected by a strong signal in the plasmin substrate spot. Plasmin can inactivate factor Xa (Pryzdial, 1999) and this reduction in factor Xa activity was observed in the microassay. Plasmin is also a potent activator of factor XU to factor XUa which can in turn convert prekallikrein to kallikrein. A high level of kallikrein substrate conversion was noted in the plasmin treated-plasma beyond that expected from plasmin-mediated conversion of the kallikrein substrate. Addition of a higher level of plasmin (2.14-1  $\mu$ M final concentration) led to significant cleavage of each substrate on the array. The microarray revealed that plasmin-mediated activation of factor XIIa resulted in kallikrein

production with sufficiently increased intrinsic pathway production of factor Xa to overcome plasmin-mediated factor Xa proteolysis to factor Xaa.

Example 5:

[0070] Programmed cell death (apoptosis) can be triggered by various cellular events such as calcium influx; oxidative stress; cytoskeletal interference; inhibitors of protein synthesis; membrane disruption or DNA disruption. Numerous chemicals induce apoptosis in specific cell types and include receptor ligands (TRAIL, FasL); ceramide-based lipids; taxol; vinblastine; cytochalasin D; topoisomerase inhibitors (etoposide); DNA cross linking agents; protein kinase inhibitors and mitochondria permeability agents (betulinic acid, rotenone).

[0071] Apoptosis occurs after activation/aggregation of surface receptors. The best studied death receptors are TNFR1 (p55 or CD120a) and CD95 (FasR or Apo1). Other death receptors include DR3 (Apo3), DR4 and DR5 (TRAIL-R2, Apo2). In cancer therapeutics, soluble TRAIL (Apo2L) can bind its receptors DR4 and DR5 to activate apoptosis in transformed cell lines but not in normal cells. Downstream signaling of death receptors is well understood, but complex. As an example, homotrimeric CD95L binds CD95 which undergoes clustering and subsequent binding of a Fas-associated death domain (FADD) protein which in turn activates Caspase 8 (FLICE). After oligomerization, Caspase 8 undergoes autoactivation which in turn activates Caspase 9. Pathways distal to TNF binding TRNRI, Apo3L binding to DR3, and Apo2L binding DR4 or DRS result in multimerization of receptors, adaptors and activation of caspases. Several caspases can proteolytically inactivate poly(ADP-ribose) polymerase (PARP) and degrade nuclear lamin which are key signatures of apoptosis.

[0072] The role of the mitochondria in apoptosis is thought to result from the ancient two-billion year old symbiosis that produced eucaryotic cells. Loss of mitochondria integrity

disrupts energy (ATP) production, triggers caspase activation and disturbs the redox potential of the cell. In caspase activation, cytochrome c (blocked by apoptosis inhibitor bcl-2) released from the mitochondria can complex with Apaf-1 and Procaspase 9 resulting in activation of Caspase 9.

[0073] Caspases (cysteiny aspartate-specific proteases) cleave protein substrates on the carboxyl terminus side of aspartate (P1 position). Positions P2, P3 and P4 also contribute to substrate specificity with P4 residues having the largest role in dictating substrate preferences among the caspases. A total of 13 distinct caspases have been identified so far. Various caspases can cleave a given fluorogenic substrate and the use of the term "a Caspase 3 substrate" does not imply that other caspases do not cleave this substrate or that Caspase 3 does not cleave other substrates. The substrate specificity of caspases has been studied through the synthesis of chromogenic and fluorogenic peptide libraries (Talanian, 1997; Thornberry, 1997). Thornberry used a 60-compound fluorogenic positional scanning library Ac-X-X-X-Asp-AMC to evaluate the specificity *in brackets* of Caspase 1 [WEHD]; Caspase 2 [DEHD]; Caspase 3 [DEVD]; Caspase 4 [(W/L)EHD]; Caspase 5 [(W/L)EHD]; Caspase 6 [VEHD]; Caspase 7 [DEVD], Caspase 8 [LETD]; Caspase 9 [LEHD] and Granzyme B [IEPD]. Similarly, various peptide aldehydes have been tested for specificity of inhibition (Garcia-Calvo, 1998) with second order rate constants  $> 10^5 \text{ M}^{-1}\text{s}^{-1}$ .

Substrate/ Inhibitor	IETD- CHO	VDVAD- CHO	DEVD- CHO	YVAD- CHO	LEHD- CHO	VEID- CHO	
	I6	I5	I4	I3	I2	I1	Blank
VEID	S1 + I6	S1 + I5	S1 + I4	S1 + I3	S1 + I2	S1 + I1	S1
LEHD	S2 + I6	S2 + I5	S2 + I4	S2 + I3	S2 + I2	S2 + I1	S2
YVAD	S3 + I6	S3 + I5	S3 + I4	S3 + I3	S3 + I2	S3 + I1	S3
DEVD	S4 + I6	S4 + I5	S4 + I4	S4 + I3	S4 + I2	S4 + I1	S4

VDVAD	S5 + I6	S5 + I5	S5 + I4	S5 + I3	S5 + I2	S5 + I1	S5
IETD	S6 + I6	S6 + I5	S6 + I4	S6 + I3	S6 + I2	S6 + I1	S6

Example 6:

[0074] A biological fluid is delivered to the chip surface as an aerosol where the fluid is a liquid sample obtained from blood; urine; saliva; biopsy; microbe or microbial preparation; virus or viral preparation; cell lysate or cell suspension or a food or agricultural product. Alternatively, the aerosol may be composed of a carrier gas such as air or nitrogen mixed with a sample gas in which are dispersed protein, viral or bacterial particles. The sample is tested for enzymatic activity where fluorogenic substrates have been arrayed in glycerol MCA substrates and enzymes.

Example 7:

[0075] A suspension of cells, DNA, total RNA or mRNA is delivered to reaction zones arrayed on a microassay chip. Individual reaction zones contain PCR primers; reverse transcriptase primers; dye-labeled oligo sequences; nucleic acid bases or fluorescent bases and enzymes such as reverse transcriptase; DNA polymerase; RNase; DNase; heat stable DNA polymerase or cleavase enzyme. The chip is subjected to heat cycles for PCR or nucleic acid synthesis or fluorescence tag incorporation or fluorescence activation of quenched entities via sequence dependent reactions. Subsequent detection can involve energy transfer between two independent fluorescent probes brought into proximity by a sequence dependent reaction dequenching of quenched molecules due to a sequence dependent reaction. Applications can include phenotypic analysis of mRNA species, genotypic analysis of DNA species and detection of single nucleotide polymorphisms (SNPs).



Example 8:

[0076] Microarrays have numerous applications in protease engineering and proteomics. A constant P1 positional scanning library of fluorogenic peptides with 19 different amino acids at the P2, P3 or P4 position (57 sublibraries) (Backes, 2000) can be accommodated by  $< 1 \text{ cm}^2$  of microarray with minimal usage of reagents. An entire scanning fluorogenic library (Harris, 2000) with 19 to 20 different amino acids in the P1- P4 positions ( $< 1200$  spots) could be accommodated on a 1"x3" slide well within the capability of glycerol spotting and aerosol deposition technology. In this example, a single protease is applied to individual fluorogenic substrates arrayed on the chip from a positional scanning library. Conversion of substrates and substrate specificity can be determined on a single microassay chip. Also, a combinatorial library of fluorogenic peptides where the identity of each amino acid in each position is well-established can be employed on a microassay chip.

[0077] Although the invention has been described above with reference to particular materials and methods, the invention is only to be limited insofar as is set forth in the accompanying claims.

## I CLAIM:

1. An assay device, comprising an array having both a planar surface and a configuration of reaction loci thereon, with each of said loci consisting essentially of at least one peptide or protein at least substantially suspended or dissolved in a hydrophilic carrier for said peptide or protein.
2. The assay device of claim 1, wherein said planar surface further comprises a nonporous chip or slide.
3. The assay device of claim 1, wherein said nonporous chip or slide includes a component selected from the group consisting of silicon; glass; silica; quartz; polystyrene and polyalkylene polymer.
4. The assay device of claim 1, wherein said configuration of reaction loci is that of a rectangular grid.
5. The assay device of claim 1, wherein said hydrophilic carrier is selected from the group consisting of saccharides, alkylene diols and alkylene polyols and said reaction loci measure between about 10 and 250 micrometers.
6. The assay device of claim 1, wherein said hydrophilic carrier is selected from the group consisting of dextran; pluronic acid; carbohydrates of the pentose; ribose or hexose families; polysaccharides; polyethylene glycol polymer; 1,2-ethanediol; 2,3-butanediol and 1,2,3-propanetriol (glycerol), and said reaction loci measure between about 50 and 100 micrometers.

7. The assay device of claim 1, wherein said reaction loci further comprises enzyme reaction components selected from the group consisting of cofactors; inhibitors; antibodies; activators and buffer elements.

8. The assay device of claim 1, wherein said reaction loci includes a biological molecule or fraction selected from the group consisting of proteins; peptides; nucleic acids; enzymes; antibodies; lipids; cell lysates and vesicles.

9. The assay device of claim 1, wherein said loci further comprises fluorogenic substrates, chromogenic substrates or other reporter substrates.

10. An assay system, comprising:

a set of operating instructions resident in computer software;

a set of computer-controlled dot applicators;

a computer-controlled device for sample aerosol generation;

a computer-controlled xy positioner;

a computer and operating software; and

a chamber for control of biological samples,

wherein dot applicators create reaction spots to which aerosolized sample droplets are applied for computer-enhanced assay of any reaction between the sample droplets and the dot constituents.

11. The assay system of claim 10, wherein said operating instructions send signals, via serial or parallel port, to start, to stop, to establish operating set points and to

control the subcomponents of the device, whereby one or more subcomponents may have an internal or external standing controller or driver.

12. The subcomponents of claim 11, further comprising multiple positive displacement microsyringe pumps; aerosol generating devices such as pressure nozzles, ultrasonic nozzles, ink-jet printheads, position-actuated ink-jet printheads, surface-actuated ink-jet printheads, fluid-contacting or fluid-noncontacting ultrasound transducers; gas flow meter/controller; xy positioner system and exhaust/filtration fan.

13. The assay system of claim 10, wherein said microsyringes hold 1.0 microliters to 1000  $\mu$ L of biological sample.

14. The assay system of claim 10, wherein said microsyringes deliver samples at a constant flow rate.

15. The assay system of claim 10, wherein said device for aerosol generation is an ultrasonic nebulizer.

16. The method for assaying a biological sample using a peptide or protein chip according to claim 1, comprising:

- a) selecting a planar surface;
- b) selecting a hydrophilic carrier and arraying a plurality of substrates in discrete reaction loci within aliquots of said hydrophilic carrier on said planar surface;
- c) applying an aerosolized or misted sample having a sample droplet size between about 5 and 15 micrometers to the array formed in step (b); and

d) detecting any reaction between the sample and the plurality of substrates.

17. A method for assaying a biological sample using a peptide or protein chip according to claim 1, comprising applying an aerosolized or misted sample onto said configuration of reaction loci and detecting any reaction between any constituents of the sample and said peptide or protein contained within said configuration of reaction loci.

18. A method for assaying a biological sample using a peptide or protein chip according to claim 1, comprising applying an aerosolized or misted sample onto said configuration of reaction loci using an ultrasonic nebulizer and detecting any reaction between any constituents of the sample and said peptide or protein contained within said configuration of reaction loci.

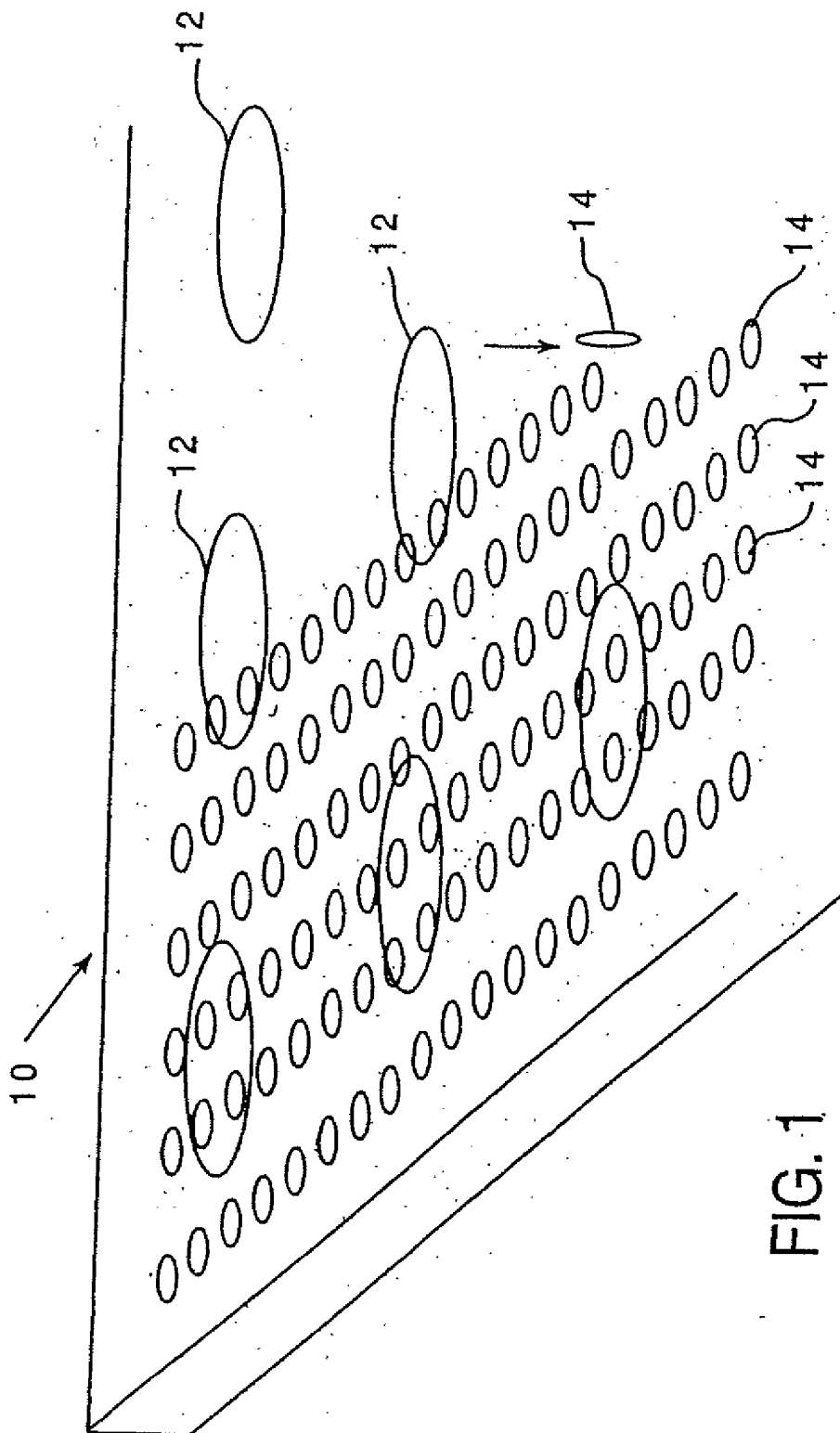
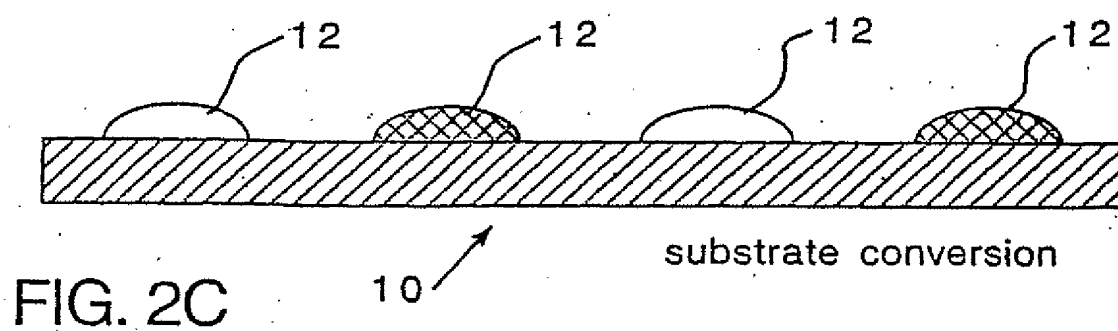
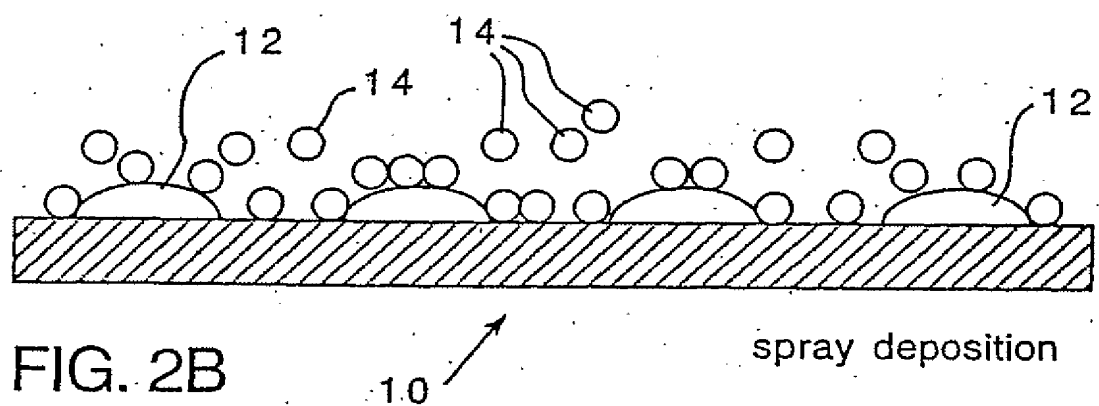
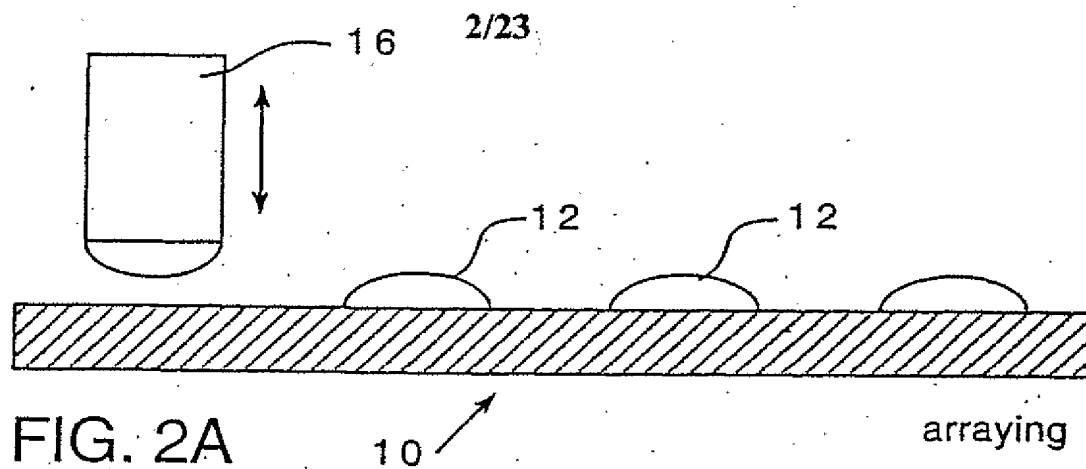


FIG. 1



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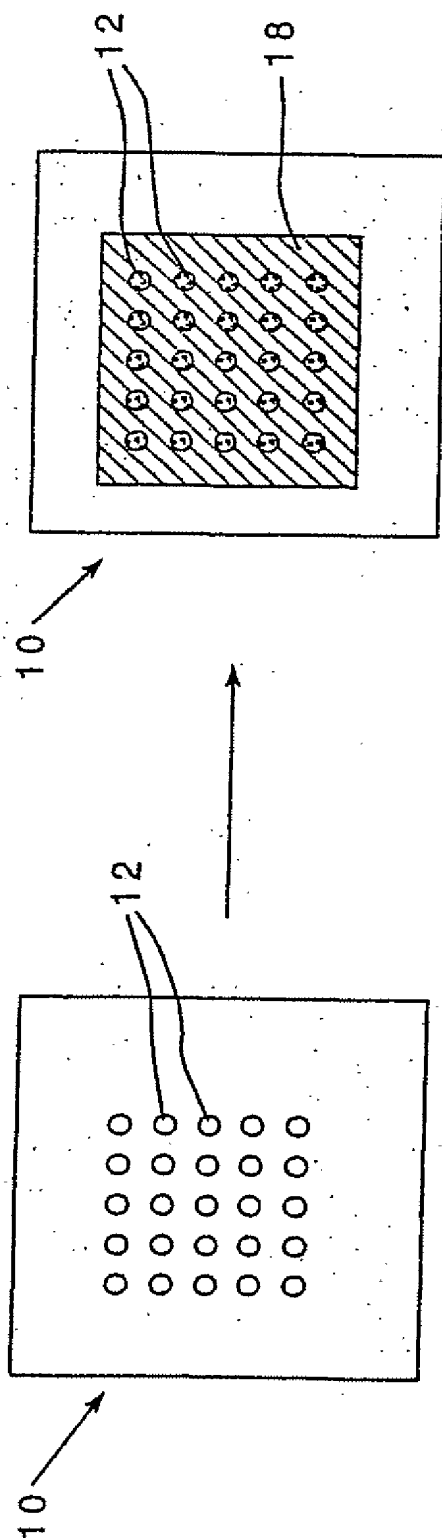


FIG. 3A

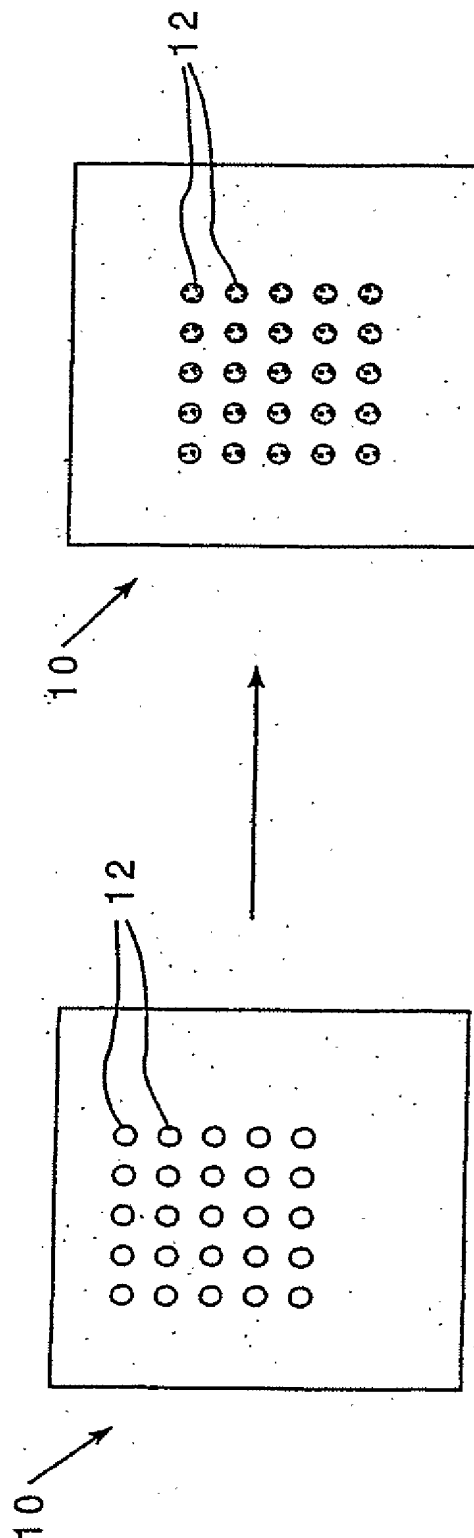
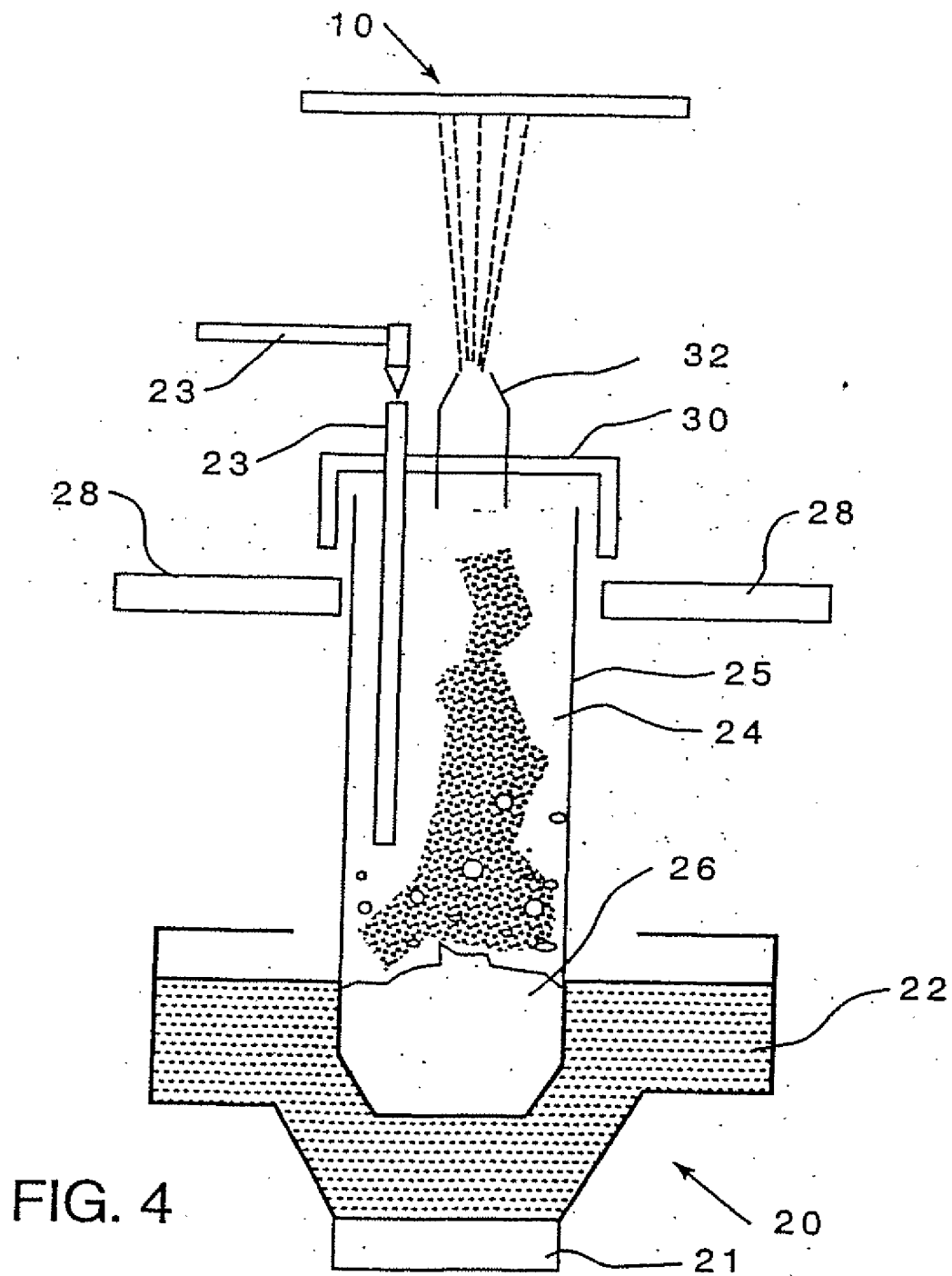


FIG. 3B



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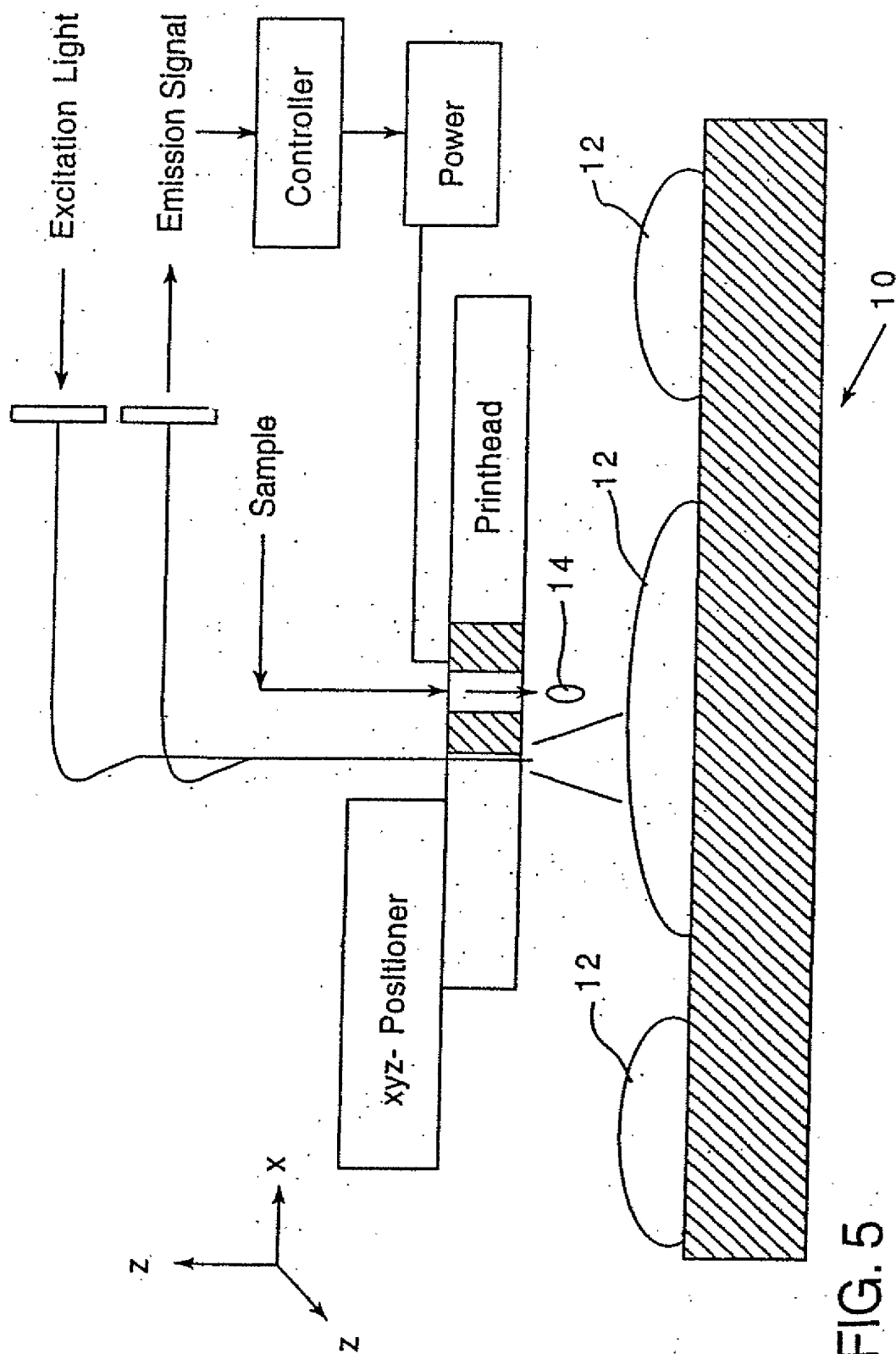


FIG. 5

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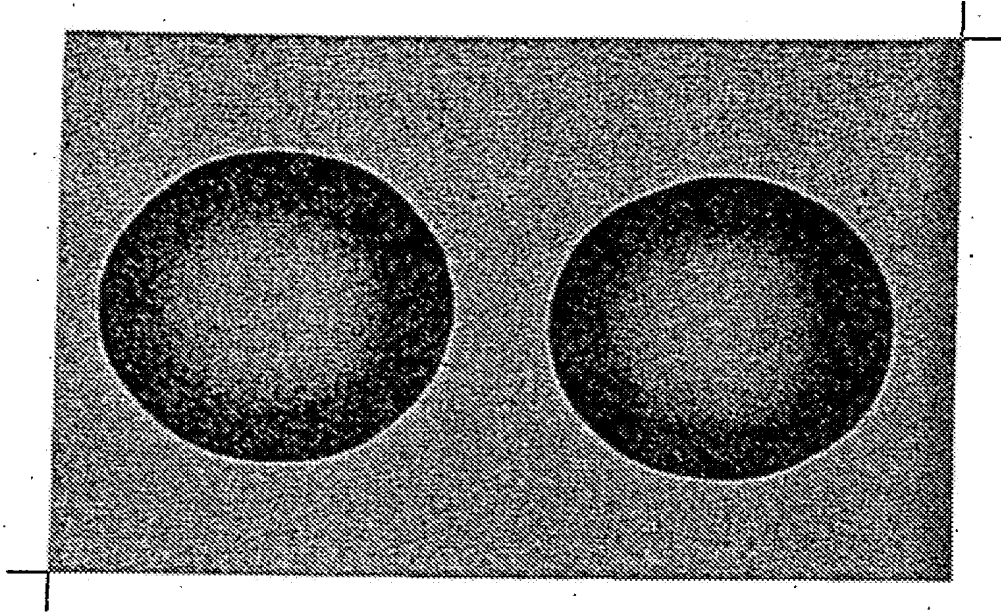


FIG. 6

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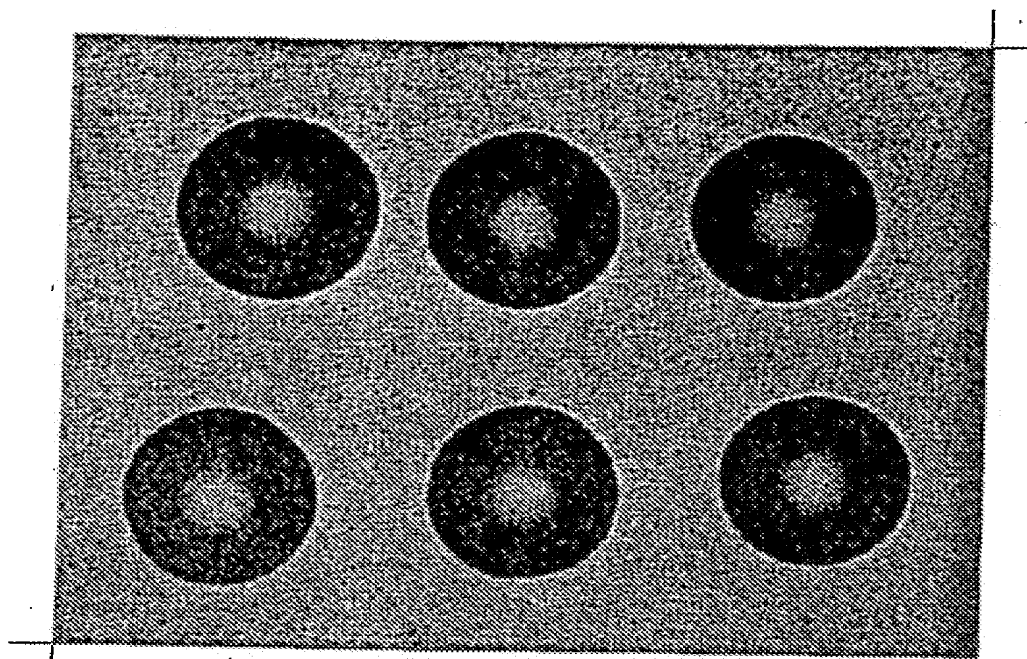


FIG. 7

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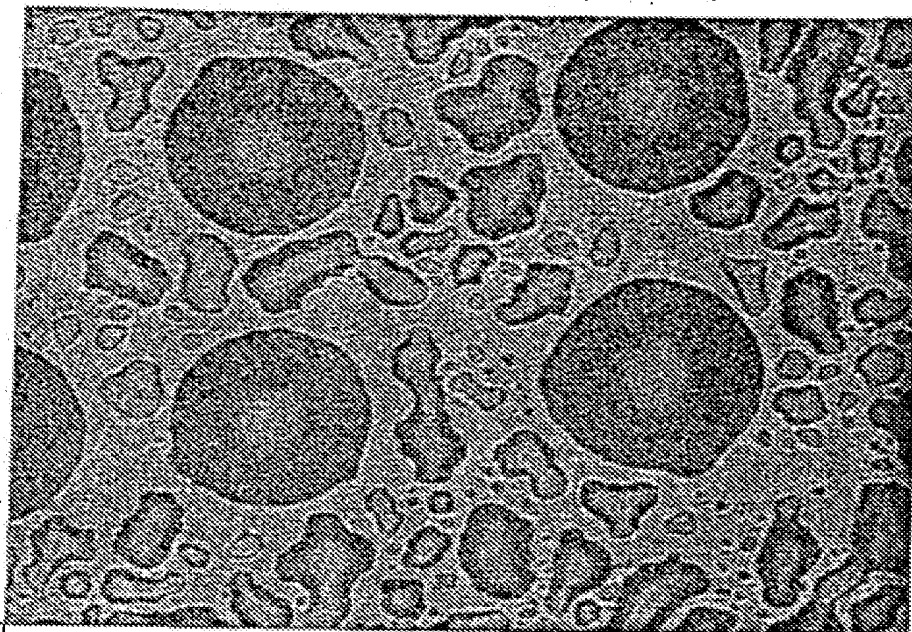


FIG. 8

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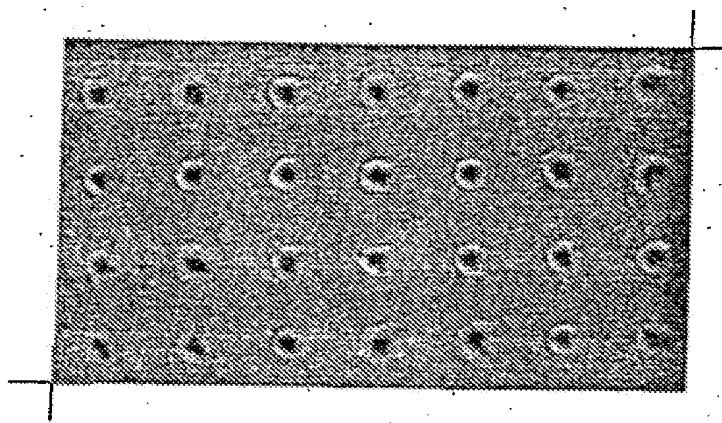


FIG. 9

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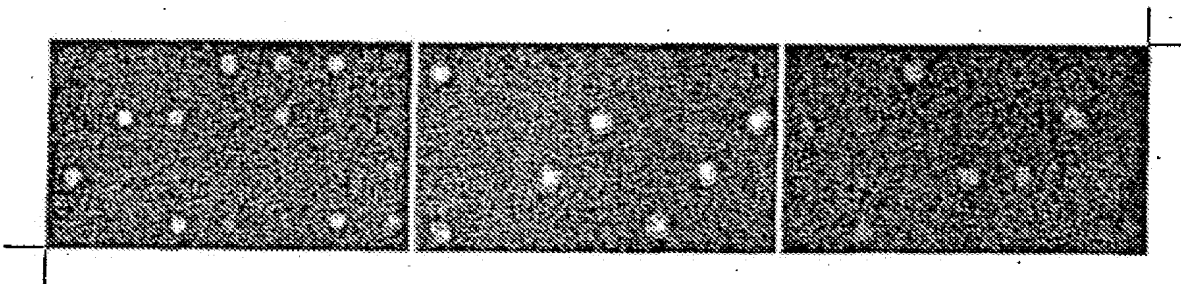


FIG. 10

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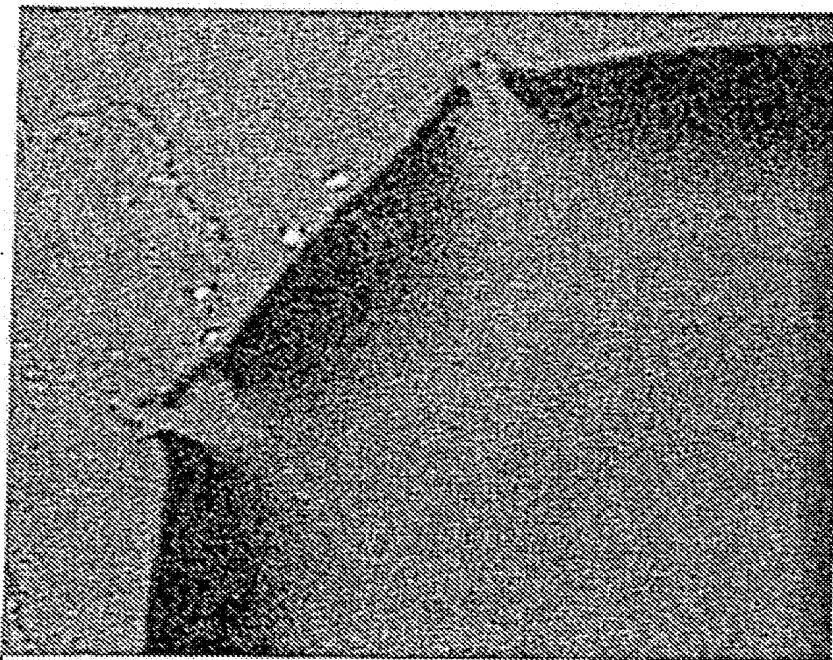


FIG. 11



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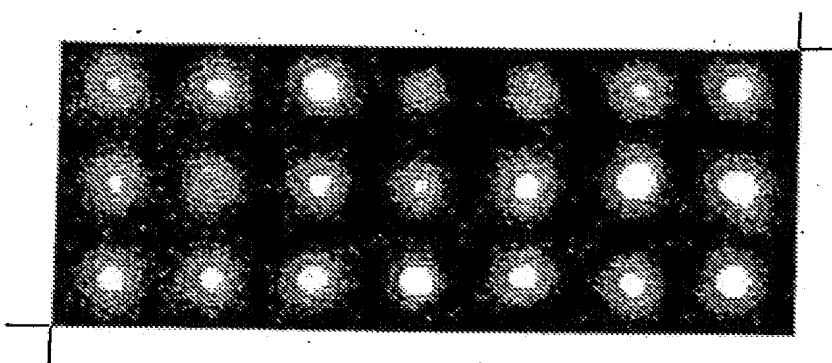


FIG. 12

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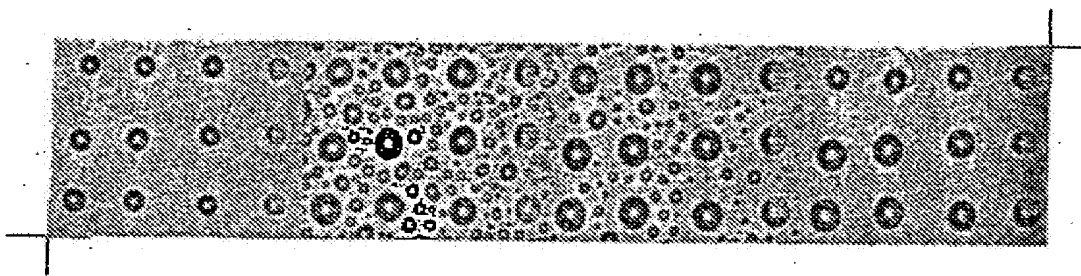


FIG. 13A FIG. 13B FIG. 13C FIG. 13D

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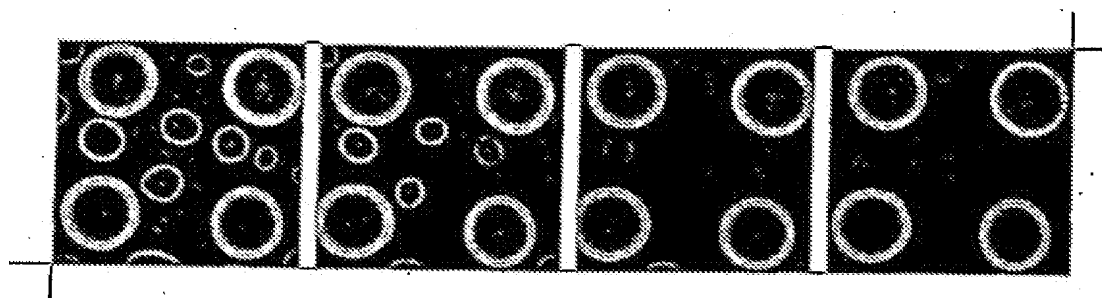


FIG. 14

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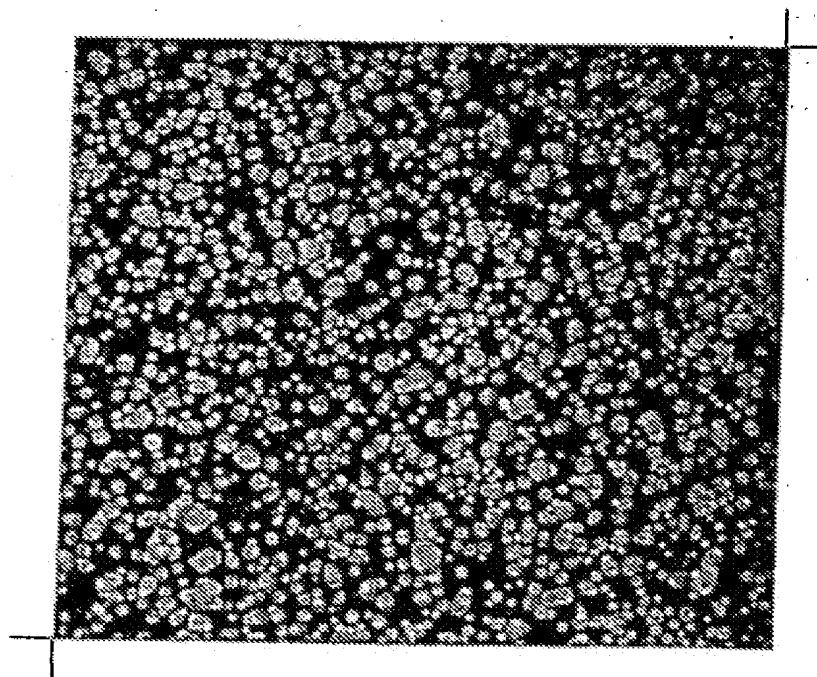


FIG. 15

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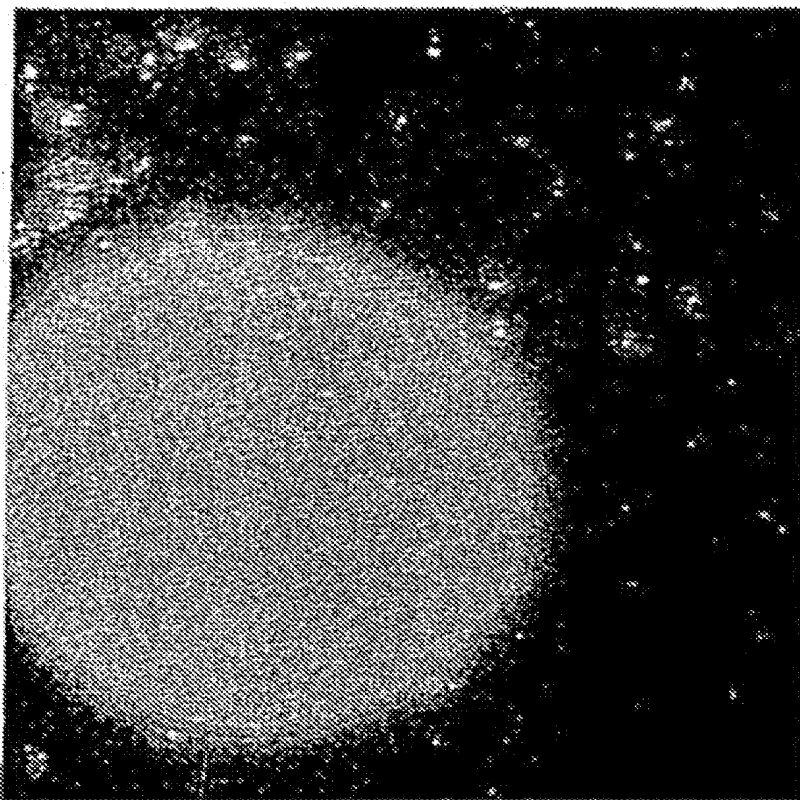


FIG. 16

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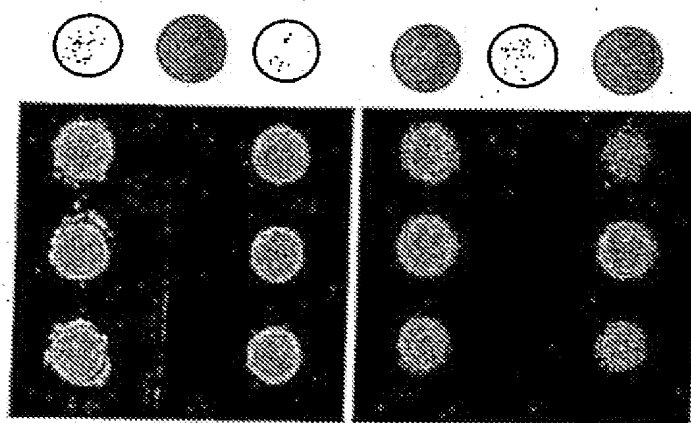


FIG. 17

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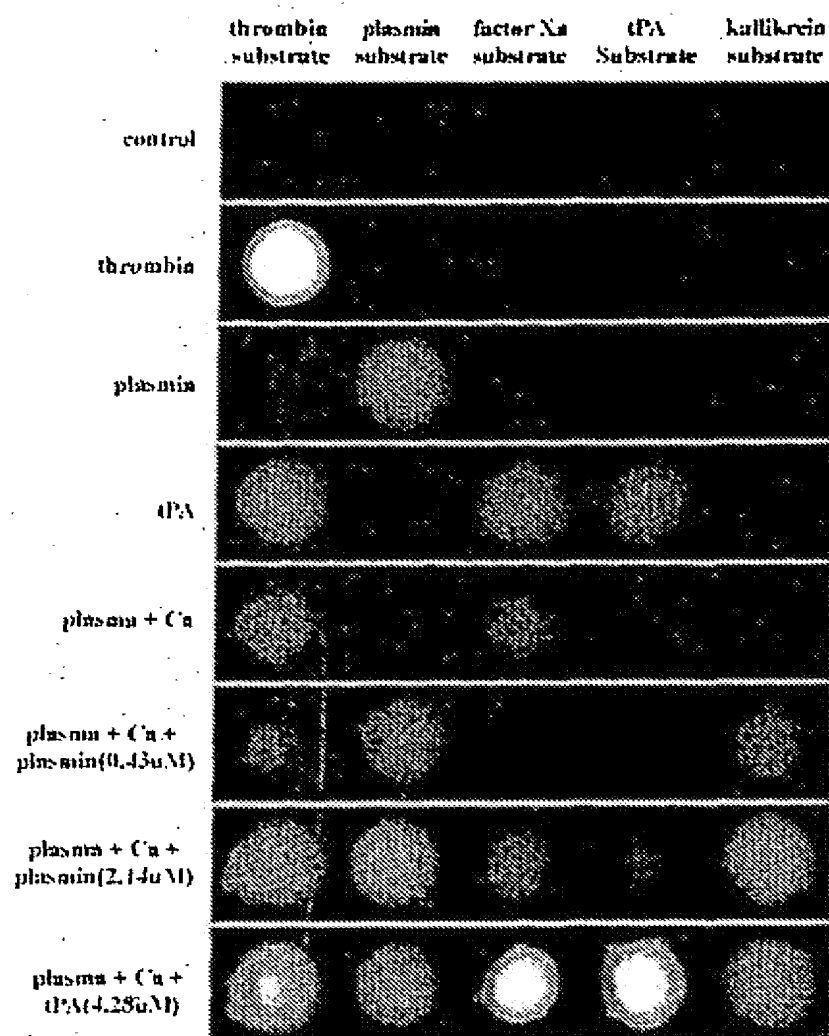


FIG. 18

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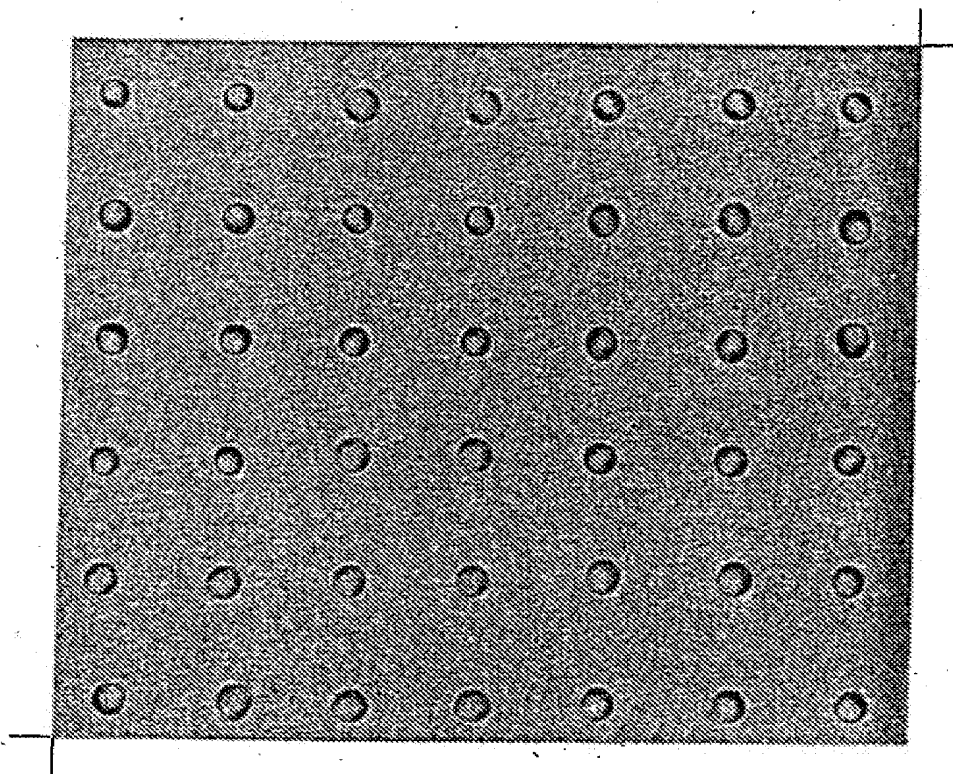


FIG. 19



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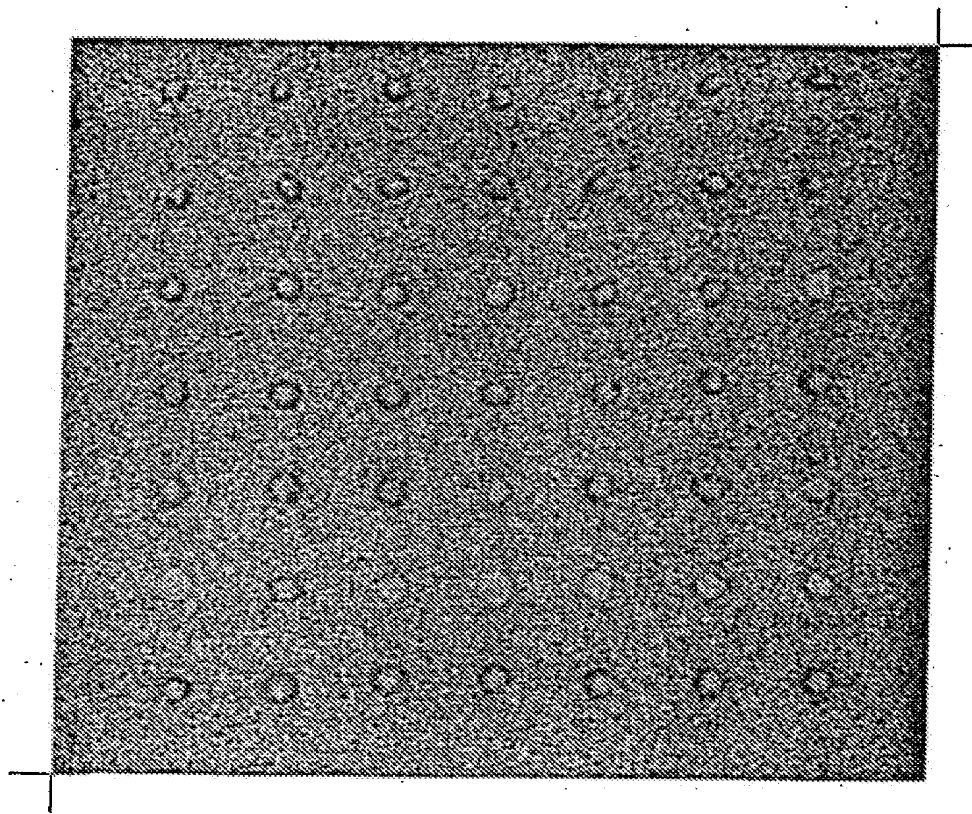


FIG. 20

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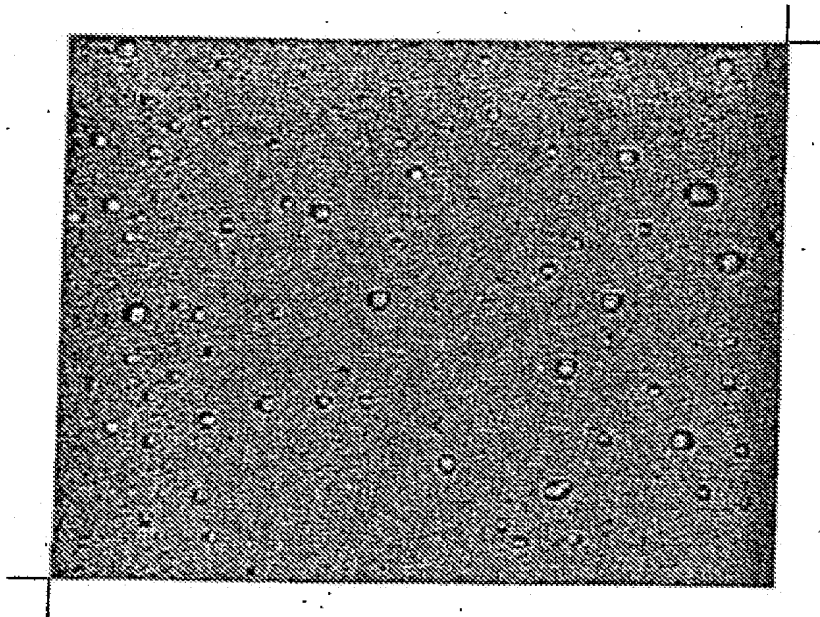


FIG. 21

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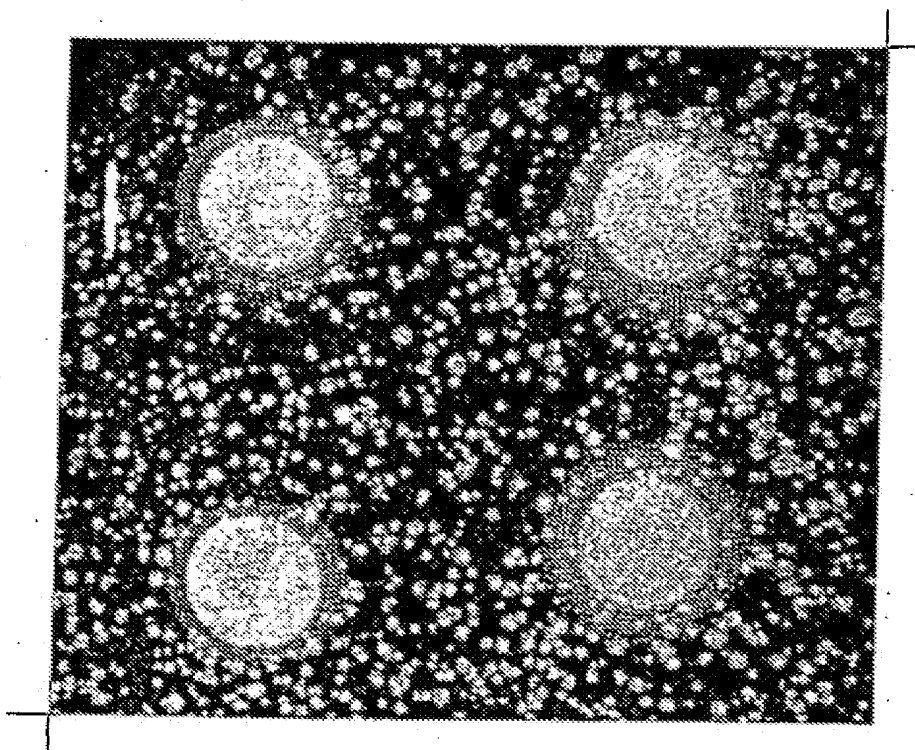


FIG. 22

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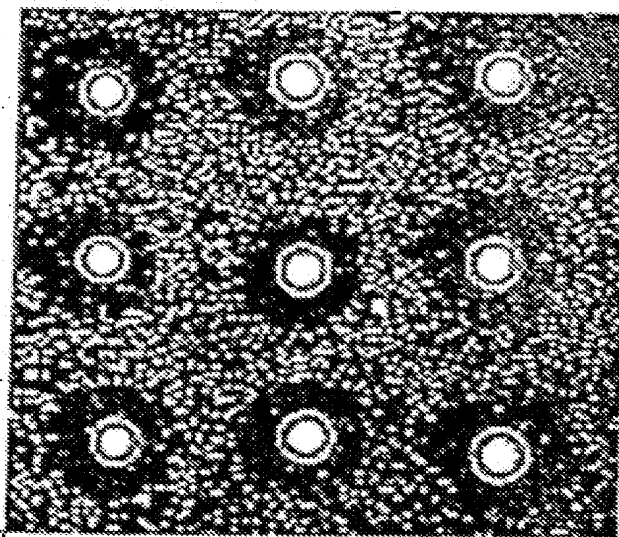


FIG. 23

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(74) Agents: JOHNSON, Barbara, E. et al.; Webb Ziesenheim Logsdon, Orkin & Hanson, 700 Koppers Building, 436 Seventh Avenue, Pittsburgh, PA 15219-1818 (US).

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(71) Applicant (*for all designated States except US*): UNIVERSITY OF PENNSYLVANIA [US/US]; 3700 Market Street, Suite 300, Philadelphia, PA 19104-3147 (US).

(72) Inventor; and

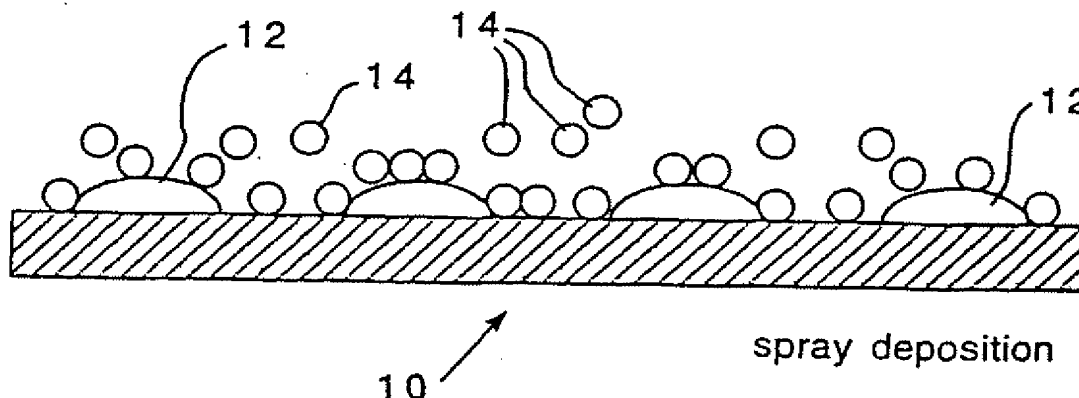
(75) Inventor/Applicant (*for US only*): DIAMOND, Scott, L. [US/US]; 610 Yale Road, Bala Cynwyd, PA 19004 (US).

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[Continued on next page]

(54) Title: PEPTIDE OR PROTEIN MICROASSAY METHOD AND APPARATUS



(57) Abstract: A peptide or protein microassay method and apparatus in which a wide variety of chromogenic or fluorogenic peptide or protein substrates of interest are individually suspended or dissolved in a hydrophilic carrier, with aliquots of each substrate being deposited in an array or microarray of reaction loci, or "dots." Each dot, therefore, provides an individual reaction vessel containing the peptide or protein of interest, to which a biological sample may be applied for assay purposes. The sample is applied to the array or microarray of dots by one of a variety of focused sample application techniques, including aerosolizing or misting of the sample, or target application of the sample, onto each dot without creating fluid channels between the dots which would cause cross-contamination.

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**(88) Date of publication of the international search report:**  
20 February 2003

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/02262

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : G01N 27/26, 33/53, 33/537, 33/543; G01D 9/00

US CL : 204/451; 435/7.1, 7.2, 288.6; 422/55, 82.05; 436/180, 518

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 204/451; 435/7.1, 7.2, 288.6; 422/55, 82.05; 436/180, 518

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,674,698 A (ZARLING et al.) 07 October 1997 (07.10.1997), entire document	1-9
Y		16
Y,P	US 6,225,047 B1 (HUTCHENS et al.) 01 May 2001 (01.05.2001) entire document	1-9
X	US 4,877,745 (HAYES et al.) 31 October 1989 (31.10.1989) entire document	10-15
Y		16-18
X,P	US 6,207,031 B1 (ADOURIAN et al.) 27 March 2001 (27.03.2001) entire document	10-15
Y,P		1-9

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search

31 May 2002 (31.05.2002)

Date of mailing of the international search report

16 DEC 2002

Name and mailing address of the ISA/US

Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703)305-3230

Authorized officer

My-Chau T. Tran

Telephone No. 703-308-0196

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/02262

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐  
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/02262

### BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-9 and 16-18, drawn to an assay device with both a planar surface and a configuration of reaction loci.

Group II, claim(s) 10-15, drawn to a computer-controlled device with a sample aerosol generation.

The inventions listed as Groups I and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature of Group I is a planar surface and a configuration of reaction loci which is not found in Group II.

The technical feature of Group II is a computer-controlled device which is not found in Group I.

Accordingly, Groups I and II are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.

CORRECTED VERSION

(19) World Intellectual Property Organization  
International Bureau



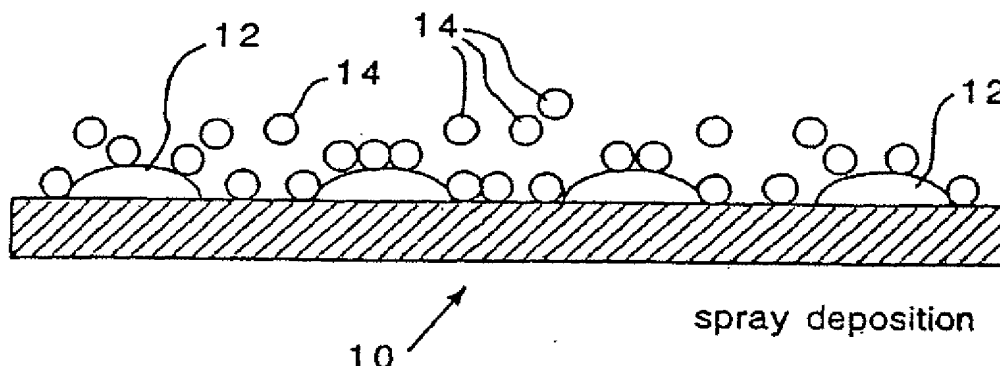
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| 60/313,377 | 17 August 2001 (17.08.2001)    | US |
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- (71) Applicant (for all designated States except US): UNIVERSITY OF PENNSYLVANIA [US/US]; 3160 Chestnut Street, Suite 200, Philadelphia, PA 19104-6283 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): DIAMOND, Scott, L. [US/US]; 610 Yale Road, Bala Cynwyd, PA 19004 (US).
- (74) Agents: JOHNSON, Barbara, E. et al.; Webb Ziesenheim Logsdon, Orkin & Hanson, 700 Koppers Building, 436 Seventh Avenue, Pittsburgh, PA 15219-1818 (US).
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